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# Glycoproteins from the Cuticle of the Atlantic Shore Crab *Carcinus maenas*: I. Electrophoresis and Western-Blot Analysis by Use of Lectins

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**Abstract.** The protein and glycoprotein content of four different neutral or acidic solvent extracts (0.5 *M* KCl, 10% EDTA, 0.1 N HCl, or 2% acetic acid) from the mineralized exoskeleton of a decapod crustacean, the Atlantic shore crab *Carcinus maenas*, were characterized by quantitative analysis of proteins, SDS-PAGE analysis, and probing with lectins on blots. The lectins used were Concanavalin A, Jacalin, soybean agglutinin, *Maackia amurensis* agglutinin II, and *Sambucus nigra* agglutinin. The results show that many proteins can be obtained from the crab cuticle without strong denaturants in the extraction medium. Many of the extracted cuticle proteins appeared to be glycosylated, bearing *O*-linked oligosaccharides and *N*-linked mannose-rich glycans. *N*-acetyl-galactosamine and *N*-acetylneuraminic acids were revealed, for the first time, as terminal residues on *N*-linked mannose-rich structures of crab cuticle glycoproteins. Sialylated glycoproteins might thus be involved in organic-mineral interactions in the calcified crab exoskeleton. The amount and variety of glycoproteins extracted with the acidic solvents are obviously different from those extracted with neutral solvents. HCl proved to be the best of the tested extraction solvents and a valuable alternative to EDTA.

## Introduction

Arthropods are characteristically invested by an exoskeleton, the cuticle. They grow by periodic molts, during which they synthesize a new cuticle and partly degrade the old one before it is shed at ecdysis (for reviews, see Stevenson, 1985; Roer and Dillaman, 1993; Goffinet and Jeuniaux, 1994). Covering different integumental regions, the cuticle acts as a protective barrier, an exchange surface, and a supporting skeleton. In decapod crustaceans, the solid cuticle of sclerites is composed of an outer thin epicuticle and three fibrous composite layers forming the procuticle (for reviews, see Neville, 1975, 1984, 1993; Goffinet and Jeuniaux, 1994; Compère, 1995). The framework of the procuticle layers consists of a helicoidal twisted plywood-like arrangement of chitin-protein microfibers (Neville, 1993). The two major procuticle layers (the pigmented layer and the principal layer) are stiffened by impregnation of the interfibrillar matrix with calcium salts, mainly calcite; the innermost layer (the membranous layer) is not mineralized and remains in contact with the epidermis throughout the anecdysial period (for reviews, see Stevenson, 1985; Roer and Dillaman, 1993; Goffinet and Jeuniaux, 1994).

The protein component of the arthropod cuticle is usually subdivided into proteins covalently bound and proteins non-covalently bound to chitin or another component (for review, see Hackman, 1984; O'Brien *et al.*, 1993). Non-covalently bound proteins are those extracted by various solvents, the differences in solubility reflecting differences in association with other cuticular components. Since most exoskeletal proteins seemed to resemble each other (Hackman and Goldberg, 1976), cuticular proteins were neglected for a long time (Willis, 1987), and those of the crustacean cuticle have not yet been characterized as extensively as those of the insect cuticle. From several recent investigations on the exoskeletal proteins of crustaceans (Skinner *et al.*, 1992; O'Brien *et al.*, 1993; Andersen, 1998, 1999; Coblentz *et al.*, 1998) and insects (Willis, 1987; Andersen *et*

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*al.*, 1995; Jensen *et al.*, 1998; Andersen, 2000), these proteins have emerged as among the most important organic constituents of the cuticle, and they are largely responsible for its mechanical properties. They are structural components of the chitin-protein microfibers and are involved in the interactions between microfibers, the interfibrillar matrix, and also the mineral in calcified exoskeletons (Andersen, 1999, 2000; Coblentz *et al.*, 1998; Willis, 1999).

In the decapod crustacean cuticle, the amount of protein does not exceed 40% of the decalcified dry weight (Welinder, 1974). Electrophoretical analyses and amino acid sequencing studies carried out on several species (*Scylla serrata*, Hackman, 1974; *Astacus leptodactylus*, Vranckx and Durliat, 1980, 1986; *Pandalus borealis*, Andersen, 1991; *Gecarcinus lateralis*, O'Brien *et al.*, 1991; Skinner *et al.*, 1992; Kumari and Skinner, 1995; Kumari *et al.*, 1995; *Homarus americanus*, Kragh *et al.*, 1997; Nousiainen *et al.*, 1998; *Cancer pagurus*, Andersen, 1999) showed that most proteins extracted from the calcified cuticles have an acidic pI and are smaller than 31 kDa. On the other hand, many recent reports suggest that acidic macromolecules—and notably glycoproteins—play a role of prime importance in the promotion and inhibition of crystal growth during calcification of diverse extracellular matrices (teeth: Akita *et al.*, 1992; bones: Hunter and Goldberg, 1993; Hunter *et al.*, 1996; mollusc shells: Wheeler *et al.*, 1988; avian eggshell: Gaudron *et al.*, 1996; sea urchin test and spicules: Berman *et al.*, 1990; Harvey *et al.*, 1995; Albeck *et al.*, 1996; for reviews, see Addadi and Weiner, 1992; Sikes *et al.*, 1994; Marsh, 1994). Few authors were interested in studying such macromolecules in crustacean exoskeletons (*Callinectes sapidus*: Shafer *et al.*, 1994; 1995; Coblentz *et al.*, 1998; *G. lateralis*: Kumari and Skinner, 1995), but they extracted glycoproteins that bear mannose-rich structures and have a relatively high apparent molecular weight (MW). These authors suggested that cuticle glycoproteins are involved in the mineralization process. In addition, Shafer *et al.* (1995) revealed that the glycosylation of the cuticular proteins of the blue crab *C. sapidus* undergoes molt-related changes concurrent with the mineralization of the cuticle after ecdysis. More recently—confirming that matrix proteins from solid cuticle of the blue crab influence *in vitro* crystallization of calcium carbonate (Gunthorpe *et al.*, 1990)—Coblentz *et al.* (1998) electrophoretically separated several crystal-associated proteins (CAPs), probably glycosylated, from extracts of the cuticle of early post-ecdysial blue crabs. They showed that these CAPs bind to calcite crystals and delay their nucleation in *in vitro* crystallization assays.

Although many studies have been devoted to the cuticular proteins of marine, freshwater, and terrestrial brachyuran decapods, conflicting results have been obtained, and close comparisons are hazardous because different species were studied and different extraction conditions used (medium composition, duration, temperature). On the other hand, because the crustacean solid cuticle is calcified, calcium salts must be solubilized prior to the extraction of proteins—especially those that are bound to the mineral. Most authors have therefore used ethylenediamine tetraacetate (EDTA), which has the advantage of promoting decalcification at neutral pH, either in the extraction medium (O'Brien *et al.*, 1991; Kumari and Skinner, 1995; Shafer *et al.*, 1994, 1995; Coblentz *et al.*, 1998), or separately, prior to the extraction (Andersen, 1991, 1999; Kragh *et al.*, 1997; Nousiainen *et al.*, 1998). According to Albeck *et al.* (1993), EDTA probably affects the aggregation state and the Ca-binding properties of the proteins, hindering their subsequent analysis; moreover, EDTA is extremely difficult to remove completely by dialysis. To extract proteins from exoskeletons, therefore, these authors recommended the use of dilute HCl, which proved more efficient than EDTA as a solvent for solubilizing proteins in an intact state from molluscan shells and sea urchin tests.

The first and main purpose of the present work is to characterize the proteins extracted from the calcified cuticle of a European marine decapod, the Atlantic shore crab *Carcinus maenas*, by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probing glycosylations on blots with various lectins. The second purpose is to compare four non-denaturing or weakly denaturing solvents (KCl, EDTA, HCl, and acetic acid) for their efficiency in solubilizing medium- and high-MW proteins and glycoproteins. The present work is also preparatory to a forthcoming paper, which tentatively aims to relate the present data to the distribution of polyanions and glycoproteins in the cuticle layers as determined by cytochemical techniques and gold-conjugated lectin probes.

## Materials and Methods

### *Biological material and preparation of cuticle extracts*

Large specimens of the Atlantic shore crab (*Carcinus maenas*), with a cephalothoracic shield exceeding 5 cm in width, were obtained from a local distributor. Anecdysial crabs were selected according to the criteria of Drach and Tchernigovtzeff (1967). The cuticle of the cephalothoracic shield was excised and carefully cleaned of adhering tissue remnants with a wet paper tissue. Samples (two sets of four) of isolated fresh cuticle, weighing 4 g each, were either crushed (first set) or ground to powder (second set), then extracted in the presence of a bactericide (10 m*M* $NaN_3$) and a protease inhibitor (1 m*M* phenylmethylsulfonylfluoride, PMSF) in one of four solvents: 0.5 *M* KCl at pH 7.0, 10% ethylenediamine tetraacetate (EDTA) at pH 7.0, 0.1 N HCl at pH 2.0, or 2% acetic acid (HAc) at pH 3.0. Extraction was carried out with stirring for 48 h at 4°C in 1 liter of solvent, which was renewed three times. The samples were enclosed in cellulose dialysis tubes (10,000 Da MW

cutoff) filled with 10 ml of solvent. The extracts were centrifuged at 14,000 × *g* for 10 min, and the supernatants were dialyzed for 40 h at 4°C against 10 m*M* $NH_4HCO_3$ containing 2.5 m*M* $NaN_3$ and 1 m*M* PMSF.

*Protein quantification assays*

Before lyophilization, the protein contents of all dialyzed extracts were measured with the bicinchoninic acid protein assay (BCA protein assay reagent kit, Pierce no. 23225), using bovine serum albumin (BSA) as the standard (Smith *et al.*, 1985; Wiechelman *et al.*, 1988). In preliminary comparisons of different protein assays, UV absorption at 280 nm, Biuret's method, and the BCA method yielded relatively close and consistent protein concentrations for crab cuticle extracts, while Bradford's technique (1976) yielded values 5 to 10 times lower. The difference is probably due to the presence of many acidic proteins in the extracts, since the Coomassie blue is known to stain such proteins weakly. Bergers *et al.* (1993) also found Bradford's method less efficient for quantifying proteins extracted from a marine diatom.

*Gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) in 15% polyacrylamide gels. Samples were solubilized by boiling for 5 min in 62.5 m*M* Tris-HCl buffer (pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue). Each well was loaded with 10 μl of a sample containing 25 to 100 μg total proteins. Electrophoretical separations were performed for 70 min at 150 V with a Hoefer SE Mighty Small II apparatus in 25 m*M* Tris-HCl-buffer (pH 6.80, 0.192 *M* glycine, 0.1% SDS). The 10-kDa Protein Ladder (GibcoBRL) was used as the molecular weight (MW) standard. Gels were stained with either Coomassie Brilliant Blue R-250 (Dzandu *et al.*, 1984) or silver (Wray *et al.*, 1981).

*Electroblotting*

Proteins were electroblotted under semi-dry conditions onto polyvinylidene difluoride (PVDF) membranes (0.2 μm, Boehringer-Mannheim) for 75 min in a Multiphor II Novablot unit (Pharmacia Biotech). Transfer buffer consisted of 25 m*M* Tris-HCl, 192 m*M* glycine (pH 8.3) made up in 15% v/v aqueous methanol. Blotting was allowed to proceed for 1 h at constant current (40 mA). To ascertain the efficiency of protein transfer, the gels were stained with Coomassie blue. The pre-stained 14,300–200,000 MW Range (GibcoBRL) was used as the protein MW standard.

*Lectin probing*

The glycoproteins immobilized on the PVDF membranes were probed with biotinylated lectins secondarily labeled with the avidin-biotinylated HRP (horseradish peroxidase) complex (Vector no. A-2004). The procedure generally followed Rohringer and Holden (1985). The secondary label was then revealed by the diaminobenzidine (DAB) staining method (Hsu and Soban, 1982) using the Sigma Fast DAB peroxidase substrate tablet set (Sigma no. D-4418). The biotinylated lectins used were Concanavalin A (Con A, Sigma no. C-2272), Jacalin (Jac, Vector no. 1155), soybean agglutinin (SBA, Sigma no. L-3395), *Maackia amurensis* agglutinin II (MAA II, Vector no. B-1265), and *Sambucus nigra* agglutinin (SNA, Vector no. B-1305). Con A essentially binds to α-mannose and α-glucose residues (α-Man, α-Glc), but not to related sugar residues such as α,β-galactose (α,β-Gal) (Goldstein *et al.*, 1965a, b; Hayat, 1993). Jac preferentially binds to galactosyl (β-1,3)*N*-acetylgalactosamine (Gal(β-1,3)GalNAc) sequences in *O*-linked oligosaccharides (Hayat, 1993; Shafer *et al.*, 1994). SBA recognizes terminal α,β-*N*-acetylgalactosaminyl (α,β-GalNAc) and α,β-galactosaminyl (α,β-Gal) residues (Lotan *et al.*, 1973; Hayat, 1993). MAA II (Knibbs *et al.*, 1991) and SNA (Broekaert *et al.*, 1984) bind to *N*-acetylneuraminic residues (NeuNAc or sialic acids) preferentially in α(2,3) and α(2,6) linkages respectively.

The probing procedure was as follows. Nonspecific binding sites on the membranes were blocked overnight at 4°C with 0.5% gelatin from coldwater-fish skin (Sigma no. G-7765) in the specific lectin buffer. For all three lectins the incubation buffer was PBS-Tween (0.01 *M* Na-phosphate, 0.15 *M* NaCl, 0.05% $NaN_3$, 0.05% Tween 20) containing 0.1% fish gelatin. This buffer was adjusted to pH 6.8 for Con A and SBA, and to pH 7.5 for Jac, MAA II, and SNA. It was supplemented with 0.1 m*M* $CaCl_2$ and 0.1 m*M* $MnCl_2$ for Con A, and with 0.1 m*M* $CaCl_2$ for SNA and Jac. The membranes were incubated for 2 h at 20°C, either with 1 μg/ml lectin for Con A and Jac, or with 5 μg/ml lectin for SBA, MAA II, SNA. They were washed six times in PBS-Tween and incubated for 2 h at 20°C with 5 μg/ml avidin-biotin peroxidase complex in PBS-Tween at pH 7.5 with 0.1% fish gelatin. After washing in PBS-Tween, the blots were washed five times in TBS (0.05 *M* Tris, 0.15 NaCl pH 7.6) and allowed to react for from 30 s to 1 min with 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride and 1 μ*M* $H_2O_2$ in TBS. They were then washed three times in TBS and three times in Milli-Q water. To test the specificity of the binding, inhibition experiments (Glass *et al.*, 1981) were performed with 0.2 *M* methyl-α-D-mannopyranoside (MMP, Sigma no. M-6160) for Con A and 0.15 *M* *N*-acetylneuraminyllactose (sialyllactose, Sigma no. A-3307) for MAA II and SNA, as lectin-binding inhibitors in the incubation media.

To determine the linkage of the carbohydrate moieties of the glycoproteins observed on the gels, extracts were treated with the enzyme *N*-glycosidase F (from *F. meningoepticum*, EC 3.2.2.18 and 3.5.1.52) under denaturing conditions be-

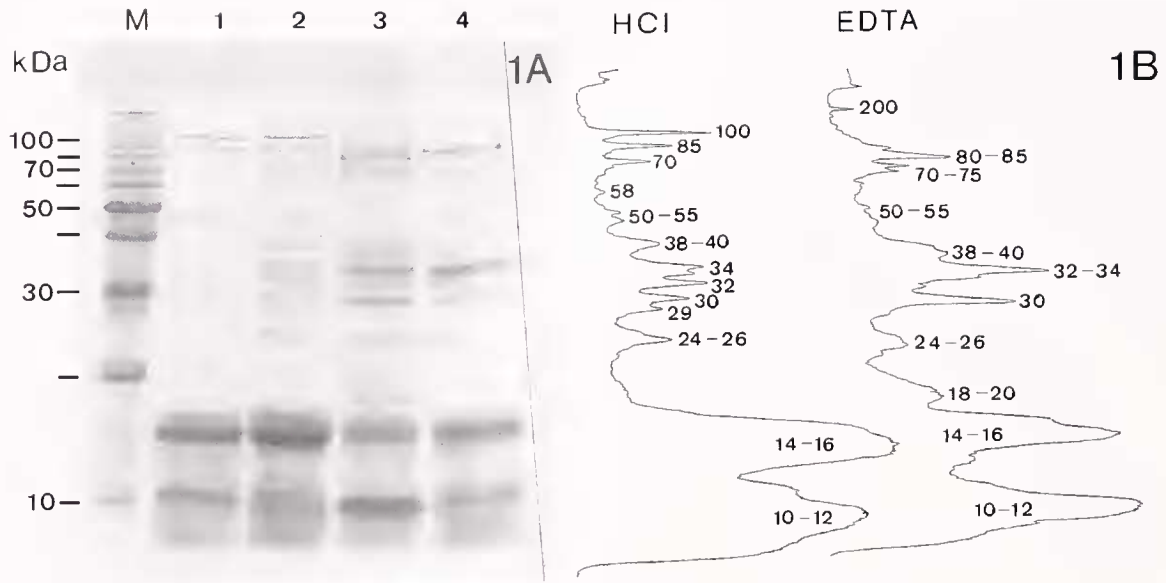


**Figure 1.** Proteins extracted from the cephalothoracic shield cuticle of anecdysial *Carcinus maenas*. Coomassie blue-stained SDS-polyacrylamide gel (15%), 50 μg protein/lane. Fig. 1A. Lane 1: 2% HAc-extract; Lane 2: 0.1 N HCl-extract; Lane 3: 10% EDTA-extract; Lane 4: 0.5 *M* KCl-extract; Lane M: Molecular Weight Marker 10 kDa Protein Ladder (GibcoBRL). Fig. 1B. Densitometric profiles of the HCl- and EDTA-extracts lanes (2 and 3).

fore SDS-PAGE, electroblotting, and lectin probing. *N*-glycosidase F specifically cleaves sugars that are *N*-linked to nonterminal asparagines of glycoproteins (Tarentino *et al.*, 1985; Maley *et al.*, 1989). Glycoproteins (100 μg) were denatured by boiling in the presence of 1% SDS (5 μl). *N*-glycosidase F buffer (45 μl; 20 m*M* Na-phosphate buffer pH 7.2, 10 m*M* $NaN_3$, 50 m*M* EDTA, 0.5% Nonidet P-40) was added, and the mixture was boiled again for 2 min. Protein deglycosylation (100 μg protein) was carried out by incubating for 20 h at 37°C in the presence of 2 units of *N*-glycosidase F (Boehringer-Mannheim).

### *Analysis of electrophoretical profiles*

Banding profiles on SDS-polyacrylamide gels and Western blots were digitized and analyzed with a Bio-Rad densitometer (model GS-670) and with the Molecular Analyst/PC program for identification of corresponding protein bands, molecular weight determinations, and densitometric profile drawing.

## Results

### *Gel electrophoresis*

Prior to gel electrophoresis, the protein contents of the extracts were estimated by the BCA method. The EDTA extract displayed the lowest protein content, ranging from 3 to 4 mg/g of initial cuticle fresh weight (FW) whatever the sample treatment (crushed or reduced into powder). The values recorded for the other extracts were from 25% to 75% higher, ranging from 5 to 6 mg/g of initial cuticle FW in the HCl extract and from 6 to 7 in the HAc and KCl extracts.

Coomassie blue and silver staining yielded quite similar electrophoretical patterns (Figs. 1 and 2), although some bands appeared with one stain and not the other, and some bands differed as to their staining intensity (detailed below). The molecular weights corresponding with the band positions were estimated from the densitometric profiles (Figs. 1B and 2B). All extracts yielded protein bands mainly in three molecular weight ranges: a few bands in a high-MW range (55–100 kDa), many in a medium-MW range (20–50 kDa), and some very thick bands in a low-MW range (10–20 kDa). The KCl and EDTA extracts displayed very similar banding profiles, with thick bands in the low-MW range, major bands in the high- and medium-MW ranges at 30 kDa, 32–34 kDa, 70–75 kDa, and 80–85 kDa, and a narrow band at 200 kDa. When extraction was done in an acidic rather than a neutral solvent, the banding pattern was different. The HCl extract displayed most of the protein bands observed with neutral solvent extracts. The only exceptions were the 200-kDa band and a weak 38-kDa band on silver-stained gels. Moreover, the HCl extract exhibited several intense additional bands at 17 kDa, 24–26 kDa, and 100 kDa. Except for a 45-kDa band that appeared in the HAc extract lane on silver-stained gels only, the banding pattern of the HAc extract closely

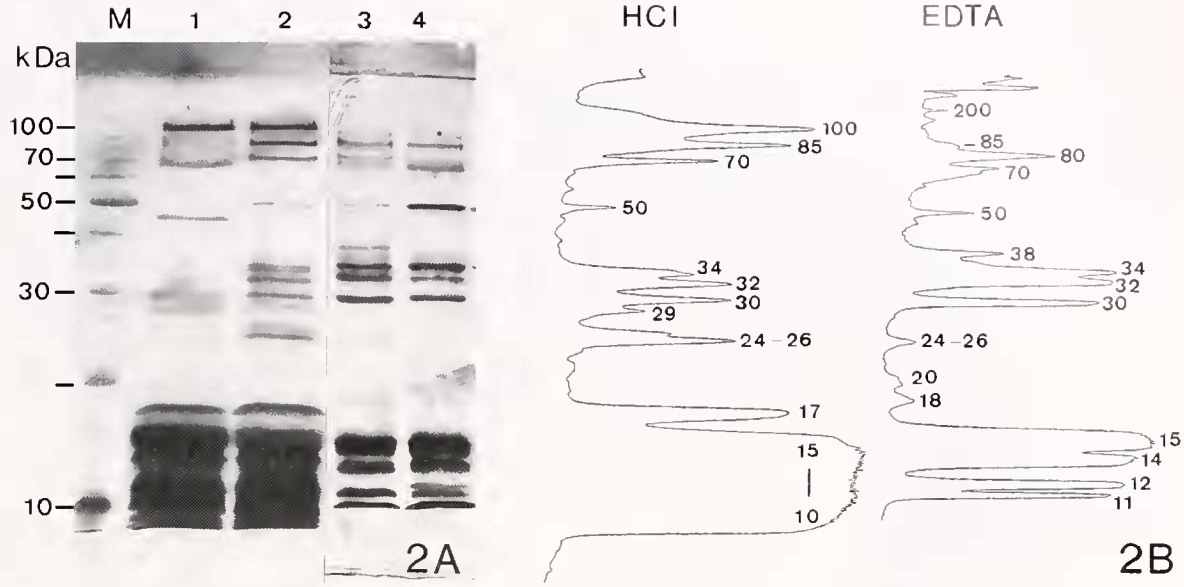


**Figure 2.** Proteins extracted from the cephalothoracic shield cuticle of anecdysial *Carcinus maenas*. Silver-stained SDS-polyacrylamide gels (15%), 50 μg protein/lane. Fig. 2A. Lane 1: 2% HAc-extract; Lane 2: 0.1 N HCl-extract; Lane 3: 10% EDTA-extract; Lane 4: 0.5 *M* KCl-extract; Lanes M: Molecular Weight Marker 10 kDa Protein Ladder (GibcoBRL). Fig. 2B. Densitometric profiles of the HCl- and EDTA-extracts lanes (2 and 3).

resembled that of the HCl extract in the low-MW ranges, but in the high- and medium-MW ranges, many bands were missing or very faint.

As mentioned above, the staining intensity of a protein band could vary drastically with the gel staining method. The 17-kDa band of the HAc and HCl extracts, for instance, was stained only by silver (Fig. 2), and the 16-kDa band that was intensely stained by Coomassie blue whatever the extract (Fig. 1) was not revealed by silver staining (Fig. 2).

*Reaction of extracted proteins with lectins*

Lectin binding revealed that most of the proteins extracted by the described procedure are glycosylated. The labeling intensity varied, however, according to the protein band and the lectin used. Conspicuous differences were again observed between neutral- and acidic-solvent extracts.

*Concanavalin A.* Of the five lectins tested, Con A labeled the greatest number of protein bands (Fig. 3). Almost all the protein bands in the high- and medium-MW ranges bound Con A, suggesting that they are glycosylated, primarily with mannose. In these ranges, only a few differences were observed between the various extracts (*e.g.*, differences in the intensity of some high-MW bands between acidic- and neutral-solvent extracts). The bands exhibiting the strongest Con A binding were mostly those staining intensely with Coomassie blue or silver. The 200-kDa band appeared to be labeled in every extract lane, even when it did not appear, or was very faint, after Coomassie or silver staining.

In the low-MW range, few of the very thick protein bands appearing on Coomassie- or silver-stained gels reacted with Con A. Moreover, obvious differences appeared between extracts. Several positive bands appearing in the range of 10–15 kDa in the lanes loaded with acidic-solvent extracts were completely missing in the EDTA-extract lane. Only two of them appeared in the KCl-extract lane, and those were very faint. A 20-kDa band accompanied the 24–26-kDa bands in all lanes. This band was faint to undetectable on Coomassie- and silver-stained gels.

Con A binding was completely inhibited by methyl-α-D-mannopyranoside (MMP) (Fig. 3A, lanes 5 and 6), except for some of the most intensely labeled bands (30–34 kDa). When the extracted proteins were treated with *N*-glycosidase F prior to separation, transferred to a PVDF membrane, and probed with Con A (Fig. 3A, lanes 7 and 8), none of the low-MW protein bands were labeled, and the high-MW protein bands appeared much fainter. This confirms that mannose-rich carbohydrates are *N*-linked to these proteins.

*Jacalin.* Like Con A, Jac bound to most of the bands in the high- and medium-MW ranges and to some low-MW bands (see Fig. 4, HCl and EDTA extracts). The reacting bands from the HCl and EDTA extracts were about the same; the only differences were a more pronounced labeling of the 65-kDa band and additional 24–26-kDa bands from the HCl extract and of the 32- and 45-kDa bands from the EDTA extract. Labeling with Jac was somewhat less intense than with Con A. Almost all of the Jac-positive bands were

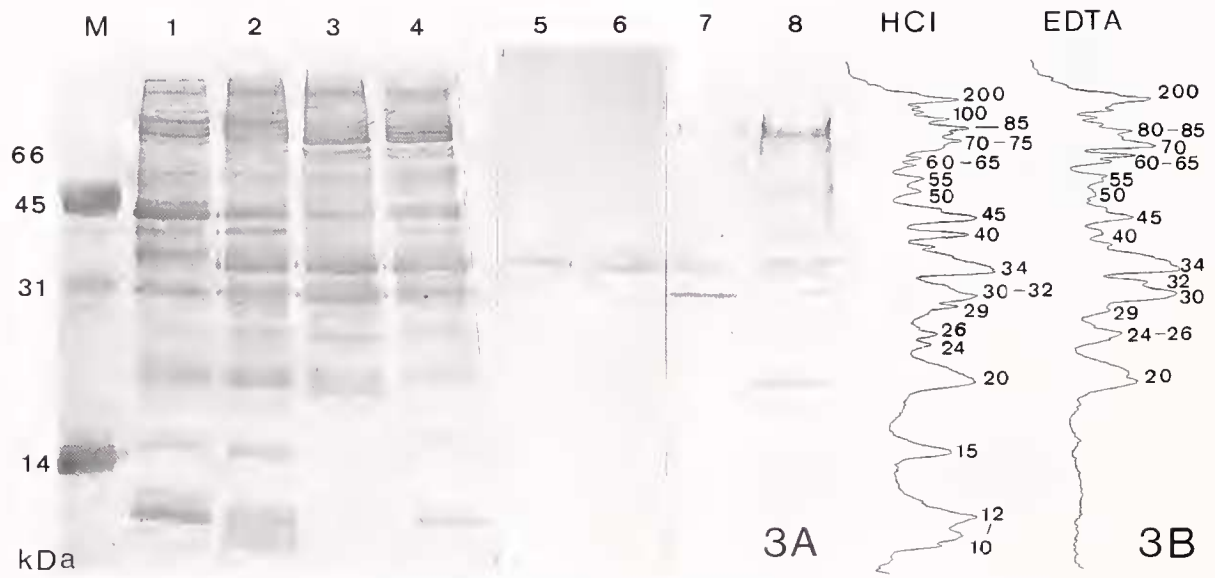


**Figure 3.** Glycoproteins of the *Carcinus maenas* cephalothoracic shield cuticle separated on SDS-polyacrylamide gels (15%, 25 μg protein/lane), electroblotted to PVDF, and probed with Con A (lanes M and 1 to 4) in the presence of MMP (lanes 5 and 6) and with previous deglycosylation by *N*-glycosidase F (lanes 7 and 8). Fig. 3A. Blotting membranes Lane M: pre-stained Molecular Weight Marker 14,300–200,000 Molecular Weight Range (GibcoBRL) with labeled 14.3-, 31- and 45-kDa bands; Lane 1: 2% HAc-extract; Lanes 2, 5, and 7: 0.1 N HCl-extract, Lanes 3, 6, and 8: 10% EDTA-extract; Lane 4: 0.5 *M* KCl-extract. Fig. 3B. Densitometric profiles of the HCl- and EDTA-extracts lanes (2 and 3).

ones previously found to react with Con A (*e.g.*, the bands at 29, 30–32, 34, 40, 45, 50, 55, and 100 kDa in both extracts; at 10, 15 and 22–24 kDa in the HCl extract; and at 80–85 kDa in the EDTA-extract), but several Con A-positive bands failed to react with Jac (*e.g.*, the bands at 12, 15, 20, 32, and 200 kDa in the HCl-extract lane; the bands at 20, 24–26, 70, and 200-kDa in the EDTA-extract lane).

Sambucus nigra *agglutinin and* Maackia amurensis *ag-*

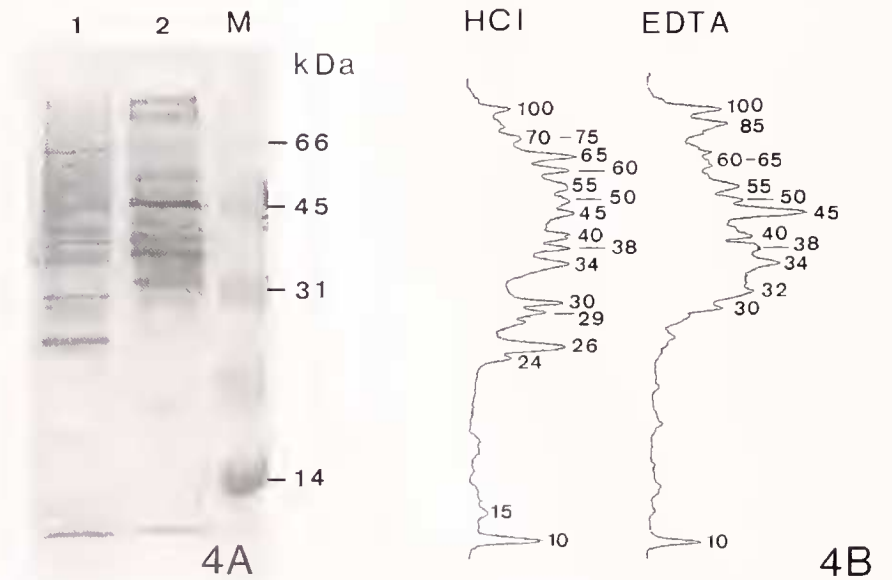


**Figure 4.** Glycoproteins of the *Carcinus maenas* cephalothoracic shield cuticle separated on 1D SDS-polyacrylamide gel (15%, 25 μg protein/lane), electroblotted to PVDF, and probed with Jac. Fig. 4A. Lane M: pre-stained Molecular Weight Marker 14,300–200,000 Molecular Weight Range (GibcoBRL) with labeled 14.3- and 45-kDa bands; Lane 1: 0.1 N HCl-extract; Lane 2: 10% EDTA-extract. Fig. 4B. Densitometric profiles.

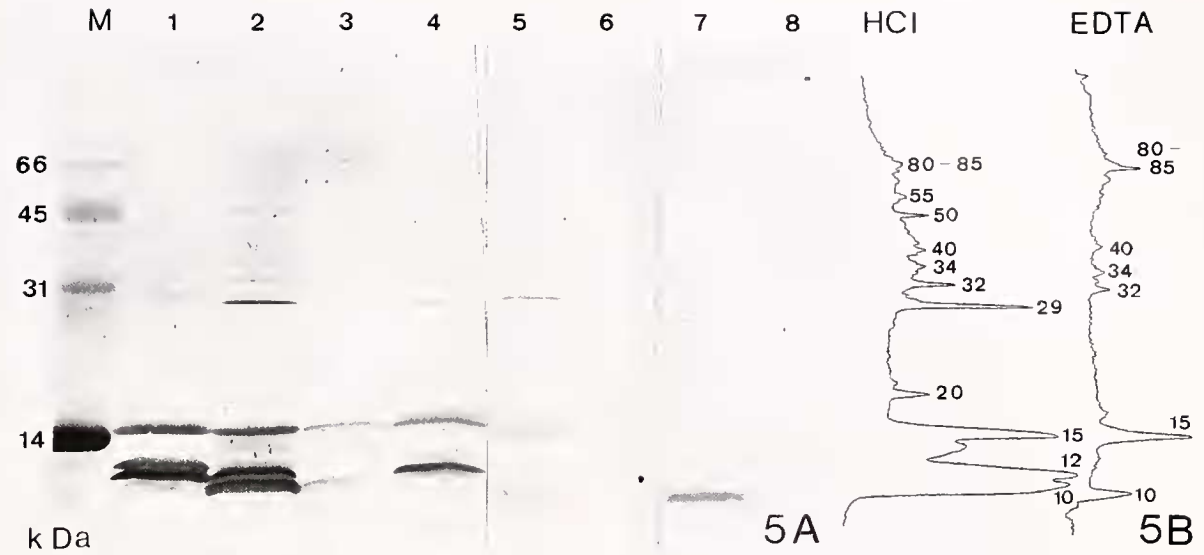


**Figure 5.** Glycoproteins of the *Carcinus maenas* cephalothoracic shield cuticle separated on 1D SDS-polyacrylamide gels (15%, 100 μg protein/lane), electroblotted to PVDF, and probed with MAA II (lanes M and 1 to 4), in the presence of sialyllactose (lanes 5 and 6) and with previous deglycosylation by *N*-glycosidase F (lanes 7 and 8). Fig. 5A. Lane M: pre-stained Molecular Weight Marker 14,300–200,000 Molecular Weight Range (GibcoBRL) with labeled 14.3- and 31-kDa; Lane 1: 2% HAc-extract; Lanes 2, 5, and 7: 0.1 N HCl-extract; Lanes 3, 6, and 8: 10% EDTA-extract; Lane 4: 0.5 *M* KCl-extract. Fig. 5B. Densitometric profiles of the HCl- and EDTA-extracts lanes (2 and 3).

*glutin II*. In contrast to Con A and Jac labeling, only a few bands were found to react with the sialic-acid-binding lectins MAA II and SNA (Figs. 5 and 6 respectively). The most intensely labeled bands appeared in the low-MW range (10–15 kDa) of the HCl and HAc extracts. Two positive bands (at 12–13 and 15 kDa) were also visible in the KCl

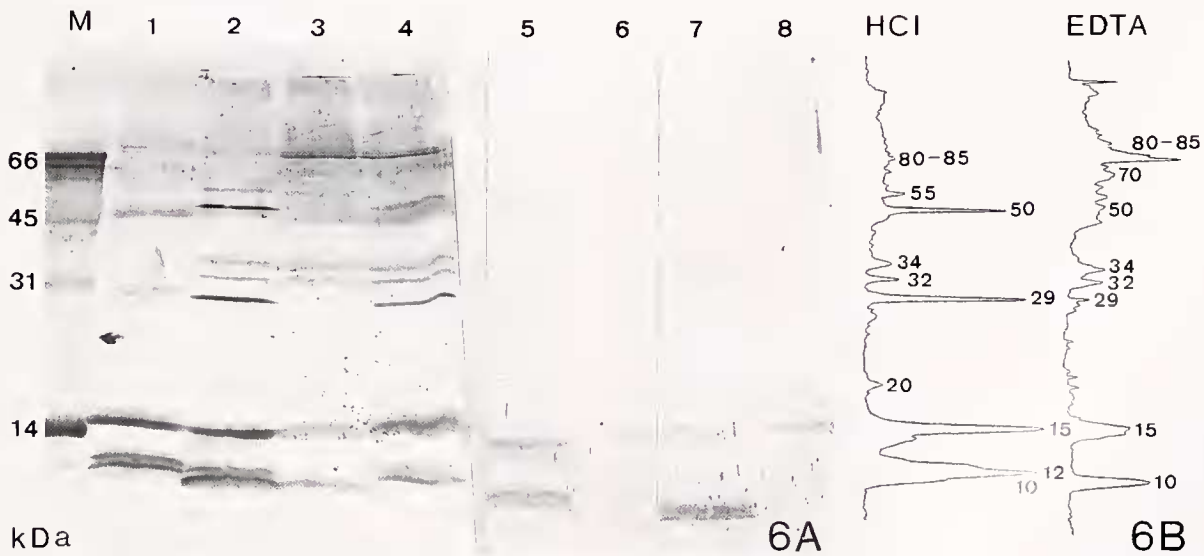


**Figure 6.** Glycoproteins of the *Carcinus maenas* cephalothoracic shield cuticle separated on 1D SDS-polyacrylamide gels (15%, 100 μg protein/lane), electroblotted to PVDF, and probed with SNA (lanes M and 1 to 4), in the presence of sialyllactose (lanes 5 and 6) and with previous deglycosylation by *N*-glycosidase F (lanes 7 and 8). Fig. 6A. Lane M: pre-stained Molecular Weight Marker 14,300–200,000 Molecular Weight Range (GibcoBRL) with labeled 14.3- and 66-kDa bands; Lane 1: 2% HAc-extract; Lanes 2, 5, and 7: 0.1 N HCl-extract; Lanes 3, 6, and 8: 10% EDTA-extract; Lane 4: 0.5 *M* KCl-extract. Fig. 6B. Densitometric profiles of the HCl- and EDTA-extracts lanes (2 and 3).

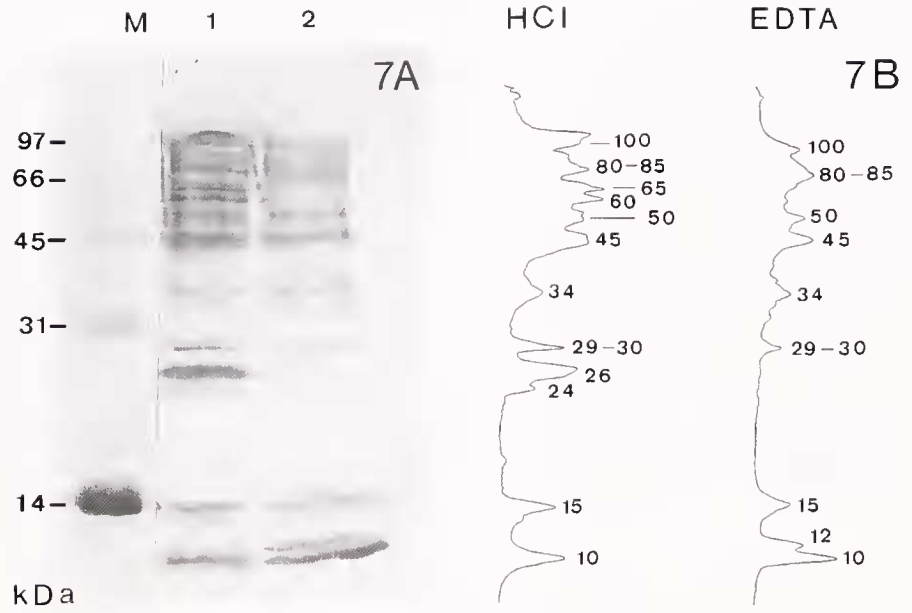


**Figure 7.** Glycoproteins of the *Carcinus maenas* cephalothoracic shield cuticle separated on 1D SDS-polyacrylamide gel (15%, 100 μg protein/lane), electroblotted to PVDF, and probed with SBA. Fig. 7A. Lane M: pre-stained Molecular Weight Marker 14,300–200,000 Molecular Weight Range (GibcoBRL) with labeled 14.3-kDa band; Lane 1: 0.1 N HCl-extract; Lane 2: 10% EDTA-extract. Fig. 7B. Densitometric profiles.

extract, but they were labeled much less intensely. They were missing or nearly undetectable in the EDTA-extract lane. In the high- and medium-MW ranges, some positive bands corresponded with ones showing a high affinity for Con A (*e.g.*, the bands at 32, 34, and 80–85 kDa in the KCl-, EDTA- and HCl-extract lanes). The HCl-extract lanes also exhibited additional, strongly positive 29- and 50-kDa bands that were missing in the EDTA-lane but present—though less intensely labeled—in the KCl-extract lane. These bands (29- and 50-kDa) reacted weakly with Con A and Jac. In the HAc-extract lane, labeling of the high- and medium-MW bands was very poor.

The intensity of SNA and MAA II labeling was significantly reduced when sialyllactose was present (Figs. 5 and 6, lanes 5 and 6), and labeling was almost completely absent when the extracts were previously deglycosylated with *N*-glycosidase F (Figs. 5 and 6, lanes 7 and 8). This suggests that the sialic acid residues detected belong to *N*-linked oligosaccharides.

*Soybean agglutinin.* As illustrated for the HCl and EDTA extracts (Fig. 7), SBA reacted with a few bands of high- and medium-MW. These were bands that also reacted with Con A, Jac, or both (*e.g.*, the bands at 29–30, 34, 45, 50, 80–85 and 100 kDa in both lanes). SBA also reacted with a few low-MW bands (in the 10–15 kDa range). The HCl extract displayed more labeled bands than the EDTA extract (additional bands at 24–26 and 60–65 kDa).

## Discussion

We have electrophoretically characterized the proteins extracted from the calcified cuticle of the Atlantic shore crab *Carcinus maenas*. The results confirm previous findings on other decapod crustaceans that many extractable proteins appear to be glycosylated. This is the first report, however, of the presence of sialic acids in crustacean cuticle extracts. We also provide new information about the type of glycosylation and the sugar residues present. Our results raise physiological questions such as the location, origin, and role of these glycoproteins in the cuticle architecture, especially as regards their interactions with the organic matrix and calcium carbonate.

In addition, the results give evidence that the nature and the related extraction capacities of the solvent used are of prime importance for subsequent analyses of proteins and glycoproteins from the calcified crustacean cuticle.

### *Extraction efficiency of the solvents*

Because much of the protein in the decapod exoskeleton is presumably associated with calcium salts, the dissolution of calcite is generally accepted as a prerequisite to the extraction of mineral-bound proteins (Gunthorpe *et al.*, 1990; O'Brien *et al.*, 1993). Thus, most of the procedures for extracting proteins from calcified cuticles have included either EDTA (0.4 *M*) together with guanidine thiocyanate (5 *M*) (O'Brien *et al.*, 1991; Kumari and Skinner, 1995; Ku-

mari *et al.*, 1995), or have involved a separate decalcification step with 0.1 *M* EDTA (Andersen, 1991) or 10% acetic acid (Kragh *et al.*, 1997; Nousiainen *et al.*, 1998; Andersen, 1999) prior to extraction by 6 *M* urea in 0.1 % trifluoroacetic acid (TFA). But with these procedures, the extraction of proteins would be mainly due to the strong denaturants (guanidine thiocyanate and urea) in the solvent. In the present study, the amount of protein extracted from the *C. maenas* cuticle by the different solvents is only a small fraction (less than 10%) of the total protein content of the cuticle, which does not exceed 40% of the decalcified dry weight, or about 15% of the cuticle dry weight (Welinder, 1974). Nevertheless, the results show that many proteins, and especially glycoproteins, can be obtained from the calcified crab carapace without strong denaturants in the extraction medium, and even without decalcification. In addition to some very intense low-MW protein bands, the electrophoretical pattern of the extracts from the *C. maenas* cuticle exhibited many protein and glycoprotein bands in the medium- and high-MW ranges (from 30 kDa up to 200 kDa), as was the case for the banding patterns recorded from the 0.1 *M* EDTA extracts (Shafer *et al.*, 1994, 1995) and the EDTA/guanidine thiocyanate extracts (O'Brien *et al.*, 1991, 1993; Kumari and Skinner, 1995) of several decapod cuticles. In contrast, in the urea extracts of previously decalcified cuticle samples from three other decapods (*Pandalus borealis*: Andersen, 1991; *Homarus americanus*: Kragh *et al.*, 1997; Nousiainen *et al.*, 1998; *Cancer pagurus*: Andersen, 1999), the proteins were mostly of low MW, and none of them appeared to be glycosylated. The molecular weight calculated by these authors from the sequence agreed with the MW determined by mass spectrometry of the intact proteins, indicating that no residue is missing. As Kragh *et al.* (1997) already implied, this indicates that substantial amounts of proteins and glycoproteins were removed during the decalcification step prior to the urea extraction. A comparison of these urea extracts (Andersen, 1991, 1999; Kragh *et al.*, 1997; Nousiainen *et al.*, 1998) with the extracts obtained with decalcifying solvents alone (Shafer *et al.*, 1994, 1995; present results) indicates that proteins preferentially extracted in neutral or acid solvents without denaturants tend to be of high MW and glycosylated, whereas proteins extracted in strongly denaturing conditions tend to be of low MW and non-glycosylated. Their amino acid sequence suggested to Nousiainen *et al.* (1998) and Andersen (1999) that such low-MW, non-glycosylated proteins could indeed be strongly bound together or to the chitin-protein microfibers, perhaps by hydrophobic interactions that would not be broken by salts or acids.

On the other hand, the results presented here demonstrated differences between samples extracted with acidic solvents and those extracted with neutral solvents. These differences were particularly obvious when comparing the HCl and the EDTA extracts, especially the lectin-binding patterns. Besides displaying the lowest total protein content, the EDTA extract had a protein-banding pattern that did not differ much from that of the KCl extract obtained without dissolving the mineral. In comparison with the HCl extract, the EDTA extract was apparently missing several bands (*e.g.*, the 17- and 100-kDa bands observed on Coomassie blue- or silver-stained gels, the Con A-labeled bands at 10–15 and 20 kDa, the Jac-labeled bands at 24–26 kDa, the SBA-labeled bands at 24–26 kDa and 60–65 kDa). Particularly poorly represented in this extract were the sialylated glycoprotein bands of low and medium MW (*i.e.*, the SNA- or MAA II-positive bands at 10–12, 15, 20, 29, and 50 kDa), even though they were also present in the KCl extract. HCl (0.1 N) thus emerged as a more efficient solvent than EDTA (10%). HCl also appeared more efficient than 2% HAc, which also seems to extract different proteins than EDTA from the cuticle of newly molted blue crabs (Coblentz *et al.*, 1998). The present electrophoretical analyses and lectin-binding patterns clearly show that many of the high-MW bands present in the HCl extract are missing in the HAc extract. The better extraction efficiency of 0.1 N HCl (pH 2.0) compared to 2 % HAc (pH 3.0) is presumably due to pronounced electrostatic repulsion between net positive charges at the low pH. With 0.1 N HCl, of course, the risk that low pH might affect or break the extracted proteins cannot be neglected. For this reason, protein extraction by 0.1 N HCl should be performed in the cold. The greater extraction efficiency of HCl compared with EDTA agrees with the conclusion of Albeck *et al.* (1993), who concluded that HCl extracts approximately twice as much protein from a molluscan shell (*Atrina rigida*) or a sea urchin test (*Paracentrotus lividus*) as does EDTA. In addition, these authors obtained better fractionation by HPLC of extract from HCl extracts than from EDTA, as well as more acidic amino acids in the HCl-extracted proteins. They proposed that traces of EDTA, extremely difficult to remove, can remain bound to the strongly ionic macromolecules and may influence their charge, conformation, and aggregation state. This could affect protein quantification, migration on gels, and subsequent detection of Ca-binding capability. Other authors (Samata and Matsuda, 1986, in Albeck *et al.*, 1993) proposed that protein might be lost during decalcification by EDTA, either due to the breakdown and leakage through the dialysis membrane, or to the binding *via* EDTA to the dialysis membrane. Such effects of EDTA would explain both the low total protein content of our EDTA extract and its low content of glycoproteins, especially those bearing sialic acids. Extraction with Tris-EDTA may also be the reason why Shafer *et al.* (1994, 1995) failed to detect MAA II- and SNA-binding glycoproteins in extracts of blue crab sclerite cuticle. Although EDTA has the advantage of dissolving the mineral at neutral pH, and thus under conditions more likely to protect the proteins, our data support the recommendation of Albeck *et al.* (1993) that mild acidic

solvents, especially dilute HCl, should be the extraction media for proteins from mineralized matrices.

*Comparison of electrophoretical patterns*

For the reasons discussed above, comparing the electrophoretical patterns obtained with cuticle protein extracts from *C. maenas* and other decapod species is limited to the results of studies in which EDTA-containing or mild acidic extraction media were used. Moreover, the apparent molecular weights of the band-forming proteins should be compared with special caution, because most of them are glycosylated. Glycosylation causes many proteins to run anomalously on SDS-polyacrylamide gels (Segrest and Jackson, 1972). As previously mentioned (O'Brien *et al.*, 1991; Kragh *et al.*, 1997), bands obtained on gels are likely to contain several proteins of similar MW, but different pI values, forming so-called "charge trains." Thus, determining whether the different types of glycans and residues identified on blots belong to a single glycoprotein or to several different ones of similar MW is impossible. Despite these limitations, comparing electrophoretical and western blot patterns of cuticular proteins in different decapod species may prove informative.

The prevalence of low-MW proteins (MW $<$ 31 kDa) appears to be a common feature in cuticle extracts from many arthropod species (Kumari and Skinner, 1993), including arachnids (Norup *et al.*, 1996). This generalization is supported by the present study. The electrophoretical patterns obtained here with *C. maenas* cuticle proteins, and the affinity of these proteins for lectins on blots, were in good agreement with previous results on other decapod species, such as *Gecarcinus lateralis* (O'Brien *et al.*, 1991, 1993; Skinner *et al.*, 1992; Kumari and Skinner, 1995) and *Callinectes sapidus* (Shafer *et al.*, 1994, 1995). This was especially true of the EDTA extract on Coomassie-stained gels and on blots probed with Con A. This suggests the presence of comparable cuticular proteins and glycoproteins in the sclerites of different crustaceans, at least among the brachyuran decapods. Remarkably, a band staining with Coomassie blue, but not with silver, was recorded at 16 kDa for *C. maenas* extracts, and at 15 kDa for *G. lateralis* extracts (O'Brien *et al.*, 1991). In *C. maenas* (present work), *Callinectes sapidus* (Shafer *et al.*, 1995), and *G. lateralis* (Kumari and Skinner, 1995) extracts, Con A recognized most of the high- and medium-MW protein bands. More precisely, major Con A-binding protein bands from *C. maenas* (*e.g.*, the bands at 20, 24–26, 30–32, 34, 45, and 50–55 kDa) coincided with those recorded from *G. lateralis* (the bands at 22, 26, 32, 34, 52, and 54 kDa). Kumari and Skinner (1995), moreover, found some low-MW, Con-A-positive bands in extracts of the procuticular layers (at 11.3, 10.2, and 9.7 kDa) similar to those observed in *C. maenas* extracts—notably the HCl extract.

Some of the above-mentioned bands in the medium-MW range might be formed by subunits of crustacyanin. Blue crustacyanin ($\beta$-form) is an oligomeric carotenoprotein that colors the carapace of decapods (Zagalsky *et al.*, 1970; Vranckx and Durliat, 1986). It is easily extracted from the cuticle by neutral and acidic solvents and dissociates irreversibly into purple subunits (40–50 kDa; $\alpha$-form) or, upon removal of the carotenoid, into subunits of about 20 kDa. Thus, several bands obtained with *C. maenas* cuticle extracts in the medium-MW range probably contain or correspond to crustacyanin subunits. This may also be true of the 54-, 50-, 48-, 44- and 42-kDa bands observed by O'Brien *et al.* (1991) with *G. lateralis* cuticle extracts. These bands are considered by the authors to be formed of specific proteins from the epicuticle (so-called the "epicuticular quintet"), which are especially prominent in extracts of the cuticle of pigmented sclerites.

*Cuticle-protein-associated glycans*

Our observation that lectins (Con A, Jac, SBA, MAA II, and SNA) recognize various blotted protein bands obtained from *C. maenas* sclerite cuticle extracts, especially HCl extracts, suggests that the glycosylated proteins contain both *N*-linked mannose-rich glycans and *O*-linked oligosaccharides. These results agree with those obtained by lectin labeling of ultrathin sections (Compère and Goffinet, unpubl. data).

The affinity of many glycoprotein bands for Jac suggests the presence of *O*-linked glycans. Jac is known to bind to the Gal($\beta$1,3)GalNAc sequences common in *O*-linked glycans. Shafer *et al.* (1994) likewise detected some Jac-positive protein bands in *Callinectes sapidus* extracts. Our results suggest that many *C. maenas* cuticular proteins of different molecular weights are *O*-glycosylated.

The presence of *N*-linked mannose-rich glycans is supported by the strong affinity for Con A of many glycoprotein bands on our blots and by the drastic reduction of Con A labeling after deglycosylation with *N*-glycosidase F. The presence of *N*-linked glycans in crab cuticular glycoproteins was previously suggested by Shafer *et al.* (1994, 1995), but not demonstrated. The present results agree with those of Kumari and Skinner (1995), who showed that Con A labeling of *Callinectes sapidus* and *G. lateralis* exoskeletal glycoproteins is sensitive to *N*-glycosidase F deglycosylation. Con A mainly recognizes both internal and terminal $\alpha$(1,2)-linked mannosyl residues, and requires two successive residues of this type to bind tightly (Ogata *et al.*, 1975; Kobata and Endo, 1992). Con A is thus known to have an affinity for many monoantennary and biantennary complex-type *N*-linked oligosaccharides. In contrast to previous results, however, our experiments suggest that many of these *N*-linked glycans bear terminal GalNAc or NeuNAc residues. Terminal $\alpha$,$\beta$ -GalNAc residues are recognized by SBA.

The similar binding patterns of MAA II and SNA, which bind to sialic acids in α(2,3) and α(2,6) linkages with different affinities, suggest that both α(2,3)- and α(2,6)-linked sialic acids are about equally represented. That these GalNAc and NeuNAc residues are present on mannose-rich structures of *N*-linked glycans is supported by several arguments. First, glycoprotein bands reacting with SBA, SNA, and MAA II correspond to ones positive to Con A. Second, the affinity of the protein bands for the three lectins and Con A is reduced by *N*-glycosidase F treatment. Finally, a mannosidase treatment (performed on tissue thin sections) abolished labeling of the cuticle layers by Con A and the three lectins (SBA, SNA, and MAA II), and it must therefore have removed, not only the mannose residues but also the terminal GalNAc and NeuNAc residues linked to them (Compère and Goffinet, unpubl. data). Kumari and Skinner (1995), in contrast, found SBA to react with only a few glycoprotein bands obtained with *G. lateralis* extracts. Shafer *et al.* (1994) detected no MAA II- or SNA-positive bands on blots from *Callinectes sapidus* and came to the conclusion that sialylated residues were absent. We, on the contrary, have detected several MAA II- and SNA-positive protein bands, especially with the HCl extract of *C. maenas* cuticle, suggesting that sialic acids may well be present as terminal sugar residues in cuticular glycoproteins.

*Prospects*

Because many of the proteins extracted from the cuticle of decapod crustaceans are glycosylated, we must ask about the role of these glycosyl residues and the location of glycoproteins in the cuticle layers. Are the detected glycoproteins constituents of the chitin-protein microfibers or of the interfibrillar matrix? Glycans might contribute, for instance, to the architecture of the cuticle or be involved in organic-mineral interactions, as reported for other extracellular matrices (Wheeler *et al.*, 1988; Berman *et al.*, 1990; Akita *et al.*, 1992; Addadi and Weiner, 1992; Sikes *et al.*, 1994; Marsh, 1994; Hunter and Goldberg, 1993; Hunter *et al.*, 1996; Gaudron *et al.*, 1996). In the crustacean cuticle, this hypothesis is reinforced by the finding that several proteins of medium and high MW extracted from the cuticle of newly molted blue crabs bind to the surface of calcite crystals, as revealed by *in vitro* crystallization assays (Coblentz *et al.*, 1998). In a report on post-ecdysial changes in the glycoproteins of the *Callinectes sapidus* cuticle, and on the lack of affinity of these glycoproteins for lectins that recognize terminal sugar residues, Shafer *et al.* (1995) hypothesized that the *N*-linked glycans of cuticular glycoproteins could be terminally sulfated or phosphated and could be involved in mineralization. The sialylated glycoproteins discovered here in *C. maenas* cuticle extracts might also interact with mineral *via* the negatively charged carboxyl groups of their sialic acids.

From our perspective, it is paramount to localize the (glyco)proteins in the cuticle. The identity of the layers from which the proteins are extracted is a question that has been tackled only by O'Brien *et al.* (1991); these authors mechanically isolated the four layers of the *G. lateralis* cuticle and processed them separately. Although some layer-specific proteins were identified, this kind of experiment is limited because the different layers interdigitate and can never be completely separated. Moreover, microorganisms on the cuticle surface and cytoplasm in the setae ducts and dermal gland ducts introduce contaminants that increase the difficulty of the problem. Consequently, a promising approach may be the ultrastructural localization of glycosyl-residues in the decapod cuticle layers by lectin labeling. This is the focus of a forthcoming paper.

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